"Nitric Oxide and Mitochondria Regulate Cytosolic Ca^{2+} Signaling in Sheared Vascular Endothelial Cells"

Undergraduate Thesis

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By

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Abstract

Vascular endothelial cell (EC) exposure to arterial-level fluid mechanical shear stress is known to cause an increase in cytosolic calcium levels ($[Ca^{2+}]_c$). The $[Ca^{2+}]_c$ increase is mediated by both extracellular Ca^{2+} influx and endoplasmic reticulum (ER)-stored Ca^{2+} release. ECs are exposed to shear stress under physiological conditions and shear stress, via the $[Ca^{2+}]_c$ increase, activates the endothelial nitric oxide synthase (eNOS) that produces nitric oxide (NO). Despite progress in understanding Ca^{2+} signaling, the exact intracellular pathways that determine Ca^{2+} homeostasis during mechanotransduction still need to be determined. In particular, it is not known whether NO has an effect on Ca^{2+} signaling and whether the mitochondria play a role in shaping the Ca^{2+} signal. The close proximity of mitochondria to the ER is thought to cause the mitochondria to experience higher local Ca^{2+} levels than the cytosol, which suggests that they could be involved in $[Ca^{2+}]_c$ signaling possibly by helping to refill the ER. To answer some of these questions, we preincubated cultured human ECs with the Ca^{2+}-sensitive fluorophore fluo-4 and discovered that shear-induced $[Ca^{2+}]_c$ shows a different spatiotemporal profile in the presence of the eNOS inhibitor L-NAME, compared to its absence. Under control conditions, 30% of ECs transiently increase their $[Ca^{2+}]_c$ within the first min of shear exposure followed by oscillations at a frequency of ~1.55/min. In the presence of L-NAME, 90% of ECs transiently increase their $[Ca^{2+}]_c$ within the first min of shear exposure, but the oscillatory response is dampened to ~1.45/min. The NO-dependent $[Ca^{2+}]_c$ response may be due to effects of NO on the cGMP-PKG-IP3R and may involve changes in the mitochondrial buffering capacity. Understanding endothelial Ca^{2+}

homeostasis is important, since deregulation of Ca^{2+} signaling is the hallmark of endothelial dysfunction and cardiovascular disease.

Acknowledgements

I would like to acknowledge the guidance by Dr. B. Rita Alevriadou, Associate Professor of BME and Internal Medicine (Cardiology) and my research advisor; Mr. Christopher G. Scheitlin, 2nd year PhD student in our lab; Dr. Randy J. Giedt, ex-PhD student, and Dr. Devi Nair, postdoctoral researcher, as well as the funding by NIH (HL106392 to B.R.A.) and AHA (to R.J.G.). I would also like to acknowledge Dr. B. Rita Alevriadou and Dr. Mark Ziolo for serving as members of my thesis defense committee.

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Lloyd CJ, Scheitlin CG, Giedt RJ, Alevriadou BR. "Endothelial Mitochondrial Ca^{2+} and Morphology Changes during a Simulated Heart Attack" presented at OSU Denman Undergraduate Research Forum (March 28, 2012)

Lloyd CJ, Giedt RJ, Scheitlin CG, Alevriadou BR."Cell Exposure to Mechanical Forces Causes Changes in Intracellular Ca^{2+} Levels" presented at OSU Fall Undergraduate Research Forum (Sept 14, 2012)

Scheitlin CD, Lloyd CJ, Giedt RJ, Alevriadou BR. "Mitochondrial Calcium Changes in Endothelial Cells Exposed to Mechanochemical Stimuli" presented at the National Biomedical Engineering Conference (Oct 24-27, 2012; Atlanta, GA)

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Chapter 1: Introduction

Intracellular calcium (Ca^{2+}) levels have been proven to play an integral role in various signaling pathways within cells. These include the promotion of the mitochondrial pathway of apoptosis as a result of cellular, and mitochondrial, Ca^{2+} overload, via activation of the permeability transition pore, a channel found on the inner mitochondrial membrane. The increase in permeability of the membrane causes release of chemicals that promote the cascade of apoptotic events, increases the production of reactive oxygen species (ROS) and, eventually, results in cell death (**Figure 1**).[1,9] In addition, mitochondrial Ca^{2+} overload has been shown to be directly involved in pathways that increase mitochondrial fission (fragmentation) and inhibit mitochondrial motility (movement) (**Figure 1**).[3,10] Due to the diverse roles of Ca^{2+} in intracellular signaling, a better understanding of the factors that modulate mitochondrial Ca^{2+} and the molecular mechanisms initiated by mitochondrial Ca^{2+} overload can offer valuable information to the scientific community and provide insight into designing drugs that can help the mitochondria, and the cell, survive under stress conditions.

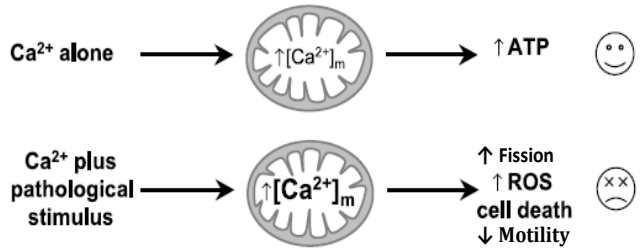

Figure 1. Effects of changes in mitochondrial Ca^{2+} inside a cell; moderate increase is beneficial, whereas Ca^{2+} overload is detrimental (modified from ref. [1])

In particular for vascular endothelial cells, extensive endothelial cell damage is known to occur in coronary arteries during/following a heart attack, a phenomenon known as endothelial dysfunction due to ischemia (period of hypoxia or anoxia/low or no flow)/reperfusion (restoration of blood flow and normal/arterial oxygen levels).[2,11] Previous work in the lab has outlined the specific mechanisms of mitochondrial ROS production that leads to mitochondrial endothelial cell damage due to ischemia/reperfusion.[5] In addition, the health/function of endothelial mitochondria, and the overall health of the cell, as a result of mechanochemical stresses has been extensively researched in our lab.[6-7] As seen below, simulated Ischemia/Reperfusion (I/RP) has the most significant effect on the shape/length of the mitochondria, compared to all other treatments, by promoting extensive fission (length shortening) (mitochondria were fluorescently labeled in endothelial cells using mitotracker green; published work form our group).[5] Based on the observation that the length of the mitochondria can be restored in the presence of antioxidants, such as N-acetyl cysteine (NAC) and oxaloacetic acid (OAA) (and in the presence of an inhibitor of fission, mitochondrial division/mitophagy inhibitor (mdivi), and a nitric oxide synthase inhibitor, L-N^G-nitroarginine methyl ester (L-NAME)), it was concluded that cellular and, specifically, mitochondrial and cellular ROS production promotes mitochondrial fission (**Figure 2**). Both increased mitochondrial ROS levels and extensive fission (as that seen during our simulated I/RP experiments) are known to lead to cell death via activation of the mitochondrial pathway of apoptosis. Determining the role of Ca^{2+} signaling in perpetuating the cell dysfunction from I/RP can offer valuable follow up information in order to build on previous work.

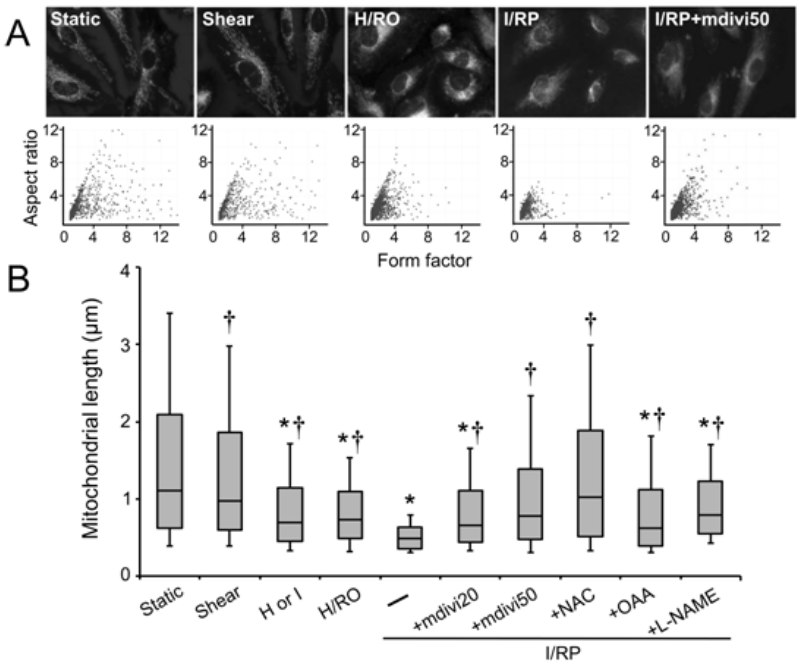

Figure 2. Endothelial cell exposure to mechanochemical treatments affects the mitochondrial morphology. (A) Fluorescence images of endothelial mitochondria (labeled with mitotracker) and calculation of mitochondrial aspect ratio (major/minor axis of an ellipse equivalent to the object) vs. form factor (perimeter^2/(4π*area)) for cells exposed to static incubation, shear stress (arterial-level flow of media over the cells), hypoxia/reoxygenation and simulated I/RP. (B) A box plot showing the mean mitochondrial length in endothelial cells exposed to static, shear, hypoxia, hypoxia/reoxygenation or simulated I/RP; the latter in the absence or presence of an inhibitor of a mitochondrial fission protein, a general and a mitochondria-specific ROS inhibitor (antioxidants) and an inhibitor of nitric oxide synthase[5]. *P<0.05 relative to static control. †P<0.05 relative to I/RP.

In order for a better understanding of the I/RP-induced endothelial dysfunction, however, it is necessary to examine the mechanisms that lead to the increased free radical production and endothelial cell apoptosis/death. Studies by others suggest that mitochondrial

Ca^{2+} overload is associated with the increased mitochondrial ROS production leading to activation of the mitochondrial pathway of apoptosis (according to **Figure 1**).[1,12]

This work is very exciting in the sense that very little is known about the role of mechanical forces, such as cellular exposure to fluid shear stress by itself or in the context of I/RP, on cytosolic Ca^{2+} ($[Ca^{2+}]_c$) signaling. Additionally, little is known about the role of nitric oxide (NO) and the regulating of intracellular calcium signaling. Endothelial nitric oxide synthase (eNOS) is activated when shear stress is applied to endothelial cells, resulting in an increase in NO concentration within the cell. An understanding of NO's contribution to intracellular Ca^{2+} signaling dynamics could give a more complete picture of the effect of shear stress on Ca^{2+} signals. Lastly, it has been postulated that, due to their close proximity to the endoplasmic reticulum (ER), mitochondria could play a role in manipulating Ca^{2+} signaling by helping to shuttle Ca^{2+} to the ER to facilitate its refilling. For this reason, an understanding of the ways in which pathological conditions that affect the mitochondria, such as I/RP injury, result in Ca^{2+} mishandling could provide valuable information in regards to the mechanisms that cause cell death/dysfunction. We hypothesize that, by understanding the Ca^{2+} response of endothelial cells to shear stress, we will be able to suggest therapeutic approaches for cardiovascular diseases with components of shear stress that result in cell dysfunction, such as I/RP injury.

Chapter II: Effect of Shear Stress on Cytosolic Calcium Signaling

Human umbilical vein endothelial cells (HUVECs) were preincubated for 2 hours with M199 (Sigma-Aldrich). During the last 20 minutes of preincubation the HUVECs were then incubated with 1 μM of Fluo-4, a cytosolic Ca^{2+} ($[Ca^{2+}]_c$) sensitive fluorescent dye. The cells were then seeded on a gelatin coated slide and sheared in a parallel plate flow chamber using a gas tight syringe and pump or a circulating flow loop to force media through the chamber (**Figure 3**). The Ca^{2+} responses were imaged using a NIKON TE2000-U fluorescence microscope and timelapse videos were recorded. A MATLAB code was developed that would read the videos and label each cell as a separate region. Using the data for each region, we were able to track the $[Ca^{2+}]_c$ within each individual cell over the duration of the video. The code was also able to calculate the average intensity of fluorescence throughout the entire region imaged over the duration of the video.

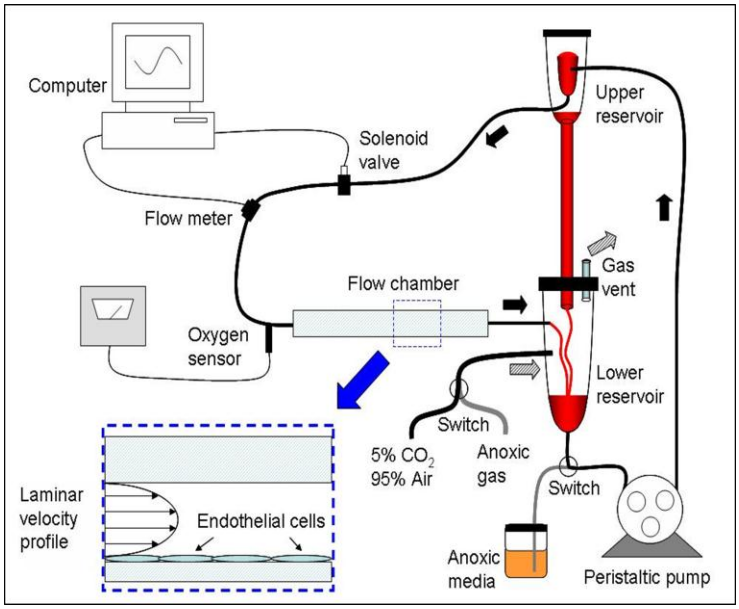

Figure 3. Flow recirculation system that exposes cultured ECs to either steady laminar shear stress or I/RP

When human umbilical vein endothelial cells were sheared at 10 dynes/cm^2 (lower arterial shear levels) they responded with $[Ca^{2+}]_c$ oscillations (**Figure 5 A and B**). This response is due to the fact that, upon the application of shear stress, the stretch activated Ca^{2+} channels (SACC) become activated and let in small amounts of Ca^{2+}. This Ca^{2+} sensitizes the inositol trisphosphate (IP_3) receptors (IP_3R) on the ER to IP_3. In addition to activating the SACC, shear stress also facilitates the production of IP_3 which causes Ca^{2+} release from the ER (citation **Figure 4**). Initially, as shear stress began, a number of the cells in the viewing area would flash, and as shear continued, they would continue to show $[Ca^{2+}]_c$ oscillations. The intensity of the subsequent peaks would continue to decrease until the oscillations ended (**Figure 5 C**).

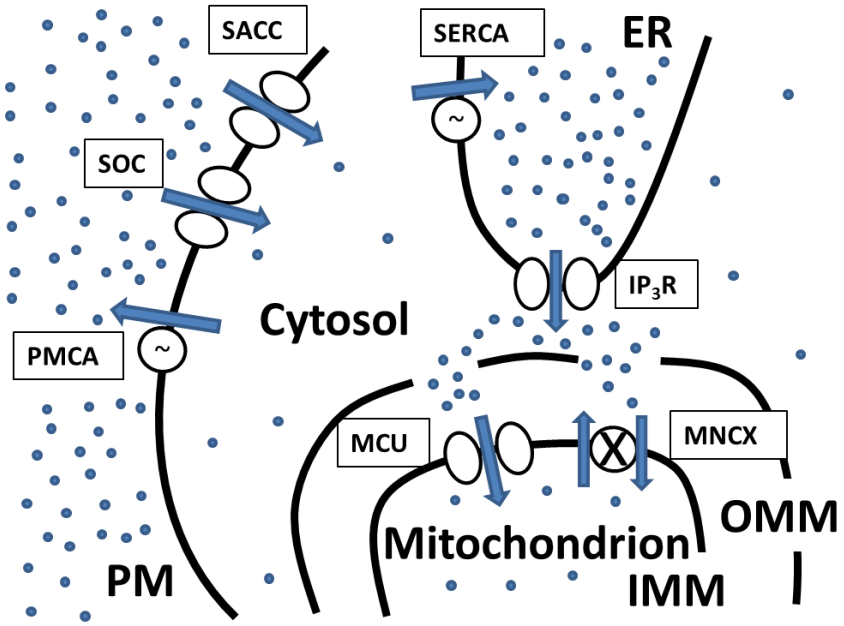

Figure 4. Schematic diagram on $[Ca^{2+}]_c$ regulation in ECs. Ca^{2+} influx from the extracellular medium occurs via Ca^{2+} channels on the plasma membrane (PM), such as the stretch-activated cation channel (SACC) and store-operated channel (SOC). Either binding of released ATP to purinergic receptors or activation of a "mechano-receptor" stimulates formation of inositol(1,4,5)-triphosphate (IP_3) which binds to IP_3 receptors (IP_3R) on the ER and triggers Ca^{2+} release from that intracellular store. Ca^{2+} regulates the IP_3R channel activity in a biphasic manner; at low Ca^{2+}, it exerts an activatory role, while it has an inhibitory effect at high Ca^{2+}. The PM and ER Ca^{2+} pumps, PMCA and SERCA, respectively, restore $[Ca^{2+}]_c$ to resting levels. Ca^{2+} released through IP_3R is transported into mitochondria via the mitochondrial Ca^{2+} uniporter (MCU) and then extruded from the organelle via the mitochondrial Na^+/Ca^{2+} exchanger (MNCX) (modified from (*6*)).

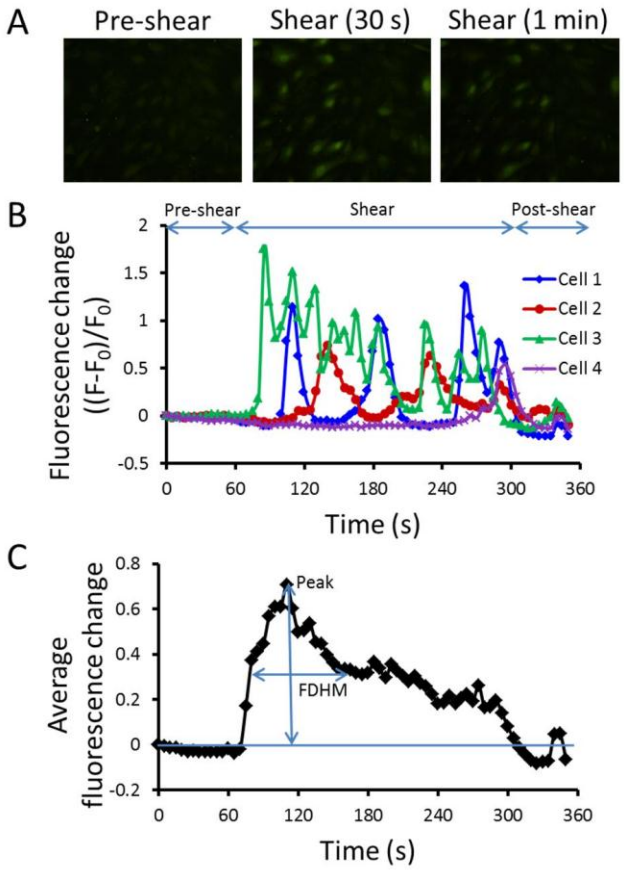

Figure 5. HUVECs exposed to shear stress demonstrate a spatially and temporally heterogeneous $[Ca^{2+}]_c$ response. (A) Fluorescence images (20x) of ECs loaded with Fluo-4 during pre-shear and following shear initiation (30 s and 1 min of shear). (B) Time course of normalized change in fluorescence during 1 min pre-shear, 4 min shear and 1 min post-shear of 4 randomly selected cells from the field of view shown in A. (C) Time course of average normalized fluorescence change (over all the cells in the field of view shown in A). Lines indicate the peak and full duration half maximum (FDHM) of the average $[Ca^{2+}]_c$ signal.

Chapter III: Effect of Nitric Oxide on Cytosolic Calcium Signaling

Experiments to determine the effect of NO on $[Ca^{2+}]_c$ signaling were performed using the same methods described in Chapter II. As opposed to simply preincubating the HUVECs in M199, however, 0.5 mM of L-N^G-Nitroarginine methyl ester (L-NAME) was also included. L-NAME is a chemical that inhibits eNOS and effectively reduces the concentration of NO produced by the cell. The M199 that was used to shear the cell monolayer also included 0.5 mM of L-NAME which prevented the production of NO upon the onset of shear.

When NO was removed from the system, the $[Ca^{2+}]_c$ increase at the beginning of shear was greatly enlarged (**Figure 5 B and C and Figure 6 C**). That means that when NO is present in the cell it effectively blunts the $[Ca^{2+}]_c$ signal. This effect can be the result of multiple possible mechanisms. The first hypothesis is that NO acts via the cGMP/PKG pathway to inhibit IP_3R and diminish Ca^{2+} release from the ER (**Figure 9**). The second possible cause for this observation is due to the fact that NO maintains mitochondrial membrane potential. Therefore, it's possible that the addition of L-NAME could cause mitochondria to lose their buffering capacity by reducing the driving force of Ca^{2+} to enter the mitochondrial matrix. Lastly, it is possible that NO interplays with the plasma membrane in a way that restricts Ca^{2+} entry via the stretch activated Ca^{2+} channels. For future experiments, however, we will choose to examine the first two mechanisms because they seem most plausible.

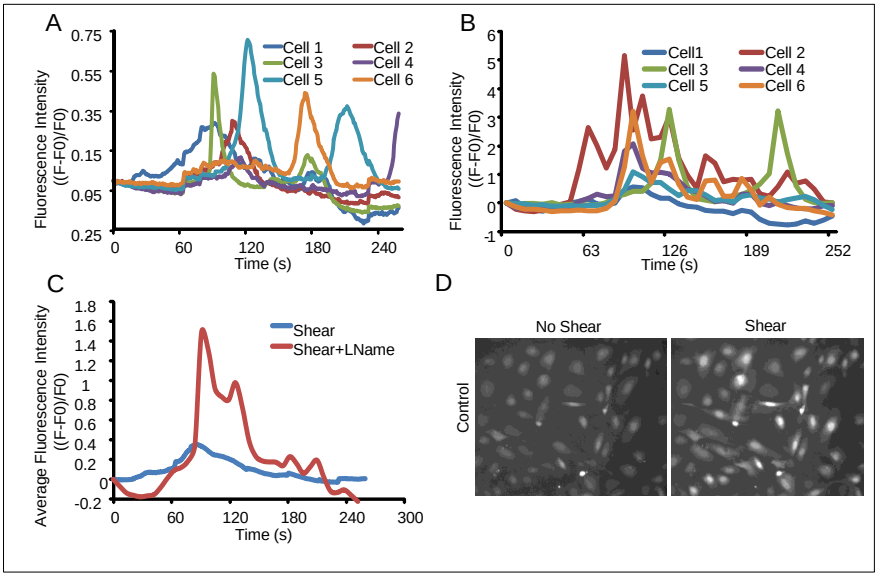

Figure 6. Effect of shear and NO on $[Ca^{2+}]c$ levels. A) Cells exposed to shear stress (10 dyne/cm^2) saw oscillations in $[Ca^{2+}]_c$ with respect to time. Each region corresponds to a single cell. B) The initial transient was increased and the oscillation frequency was decreased when the cells were preincubated with the NO inhibitor, L-Name. C) The changes in average fluorescence intensity among all the cells in the viewing region with respect to time for each condition. D) Characteristic Fluo-4 images of cells before and after exposure to shear stress.

In addition to exaggerating the $[Ca^{2+}]_c$ response at the onset of shear, removing NO from the system also increased the percentage of responding cells (**Figure 6 A**). This again can be contributed to lack of NO and its blunting effect on Ca^{2+} signaling. Removing NO also suggested a trend that the oscillation frequency and FDHM (**Figure 4 B and D**) of the initial calcium response was decreased, but until more trials are run, the difference with the control cannot be considered significant.

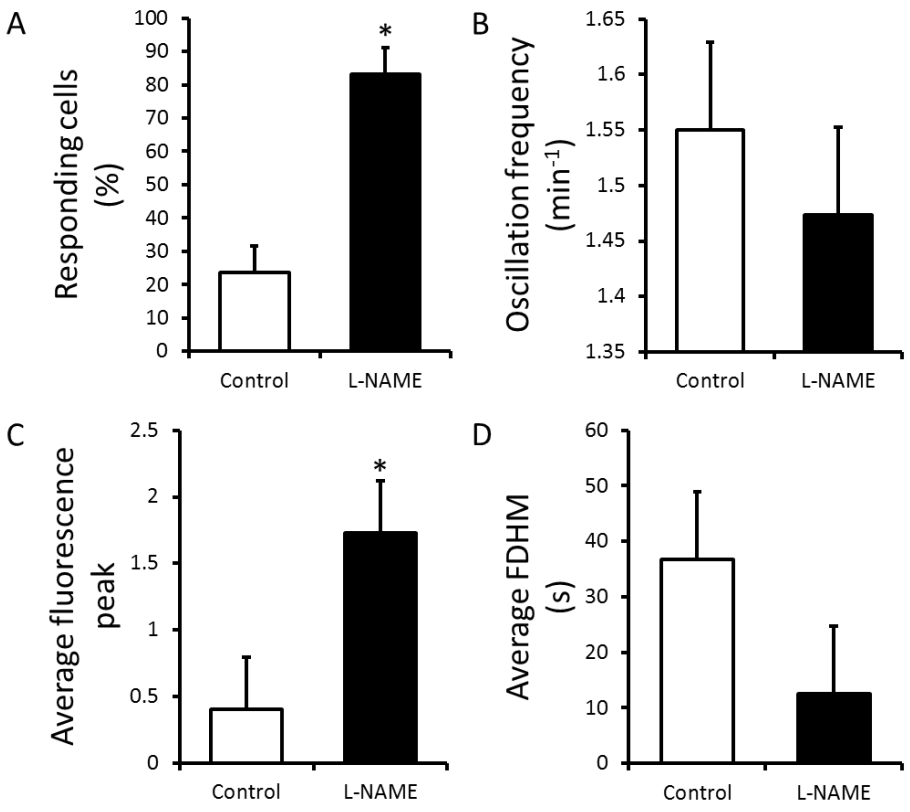

Figure 7. NO blunts the shear-induced $[Ca^{2+}]_c$ response. (A) Almost all of the L-NAME-treated ECs responded to shear with a change in their $[Ca^{2+}]_c$ (during the first min of shear exposure) in contrast to control ECs. (B) L-NAME did not change significantly the frequency of oscillations in the responding cells (during the first min of shear), but the data suggests that with more runs the data may show that oscillation frequency does decrease significantly when L-NAME is added. (C) L-NAME significantly increased the average fluorescence peak compared to control. (D) L-NAME appeared to decrease, but not significantly, the average FDHM. Data are mean±SEM for n≥4 independent experiments. *p<0.05 relative to control (n=4 tested by student's t-test).

Chapter III: Effect of mitochondria on intracellular calcium signaling

For the experiments to test the overall role of mitochondria in shaping $[Ca^{2+}]_c$ signaling, the methods were again very similar to those described in Chapter II. The difference for these experiments being that, following the 2 h of preconditioning in M199 and 20 min in Fluo-4, the cells were treated with 5 µM p-triflouromethoxyphenylhydrazone (FCCP) and 5 µM oligomycin for 2 min prior to shear. Treating with FCCP uncouples the mitochondria and dissipates the mitochondrial membrane potential, and treating with oligomycin inhibits ATP synthase. Incubating the cells with these two chemicals renders the mitochondria completely dysfunctional and unable to contribute to Ca^{2+} signaling dynamics.

Though only one experiment has been run so far, inhibiting the mitochondria within the HUVECs greatly changed the dynamics of the $[Ca^{2+}]_c$ signaling. As seen from the averaged fluorescence data, there are noteworthy differences in the fluorescent traces between the control and cells with their mitochondria inhibited (**Figure 6 A and B**). Additionally, the oscillation frequency is drastically lower in cells with their mitochondria inhibited, when compared to the control (**Figure 6 C**). If this trend repeats itself in subsequent experiments, this woud mean multiple things. This would reaffirm the hypothesis that mitochondrial handling is important in order to maintain normal $[Ca^{2+}]_c$ dynamics. This is possible due to the fact that Ca^{2+} has been shown to increase IP_3R sensitivity to IP_3. Mitochondria could play a role in reducing the local $[Ca^{2+}]_c$ seen by the ER membrane and the IP_3R. Additionally, it could mean that, due to their

close proximity to the ER, the mitochondria are required to shuffle Ca^{2+} to the ER in order to facilitate its refilling.

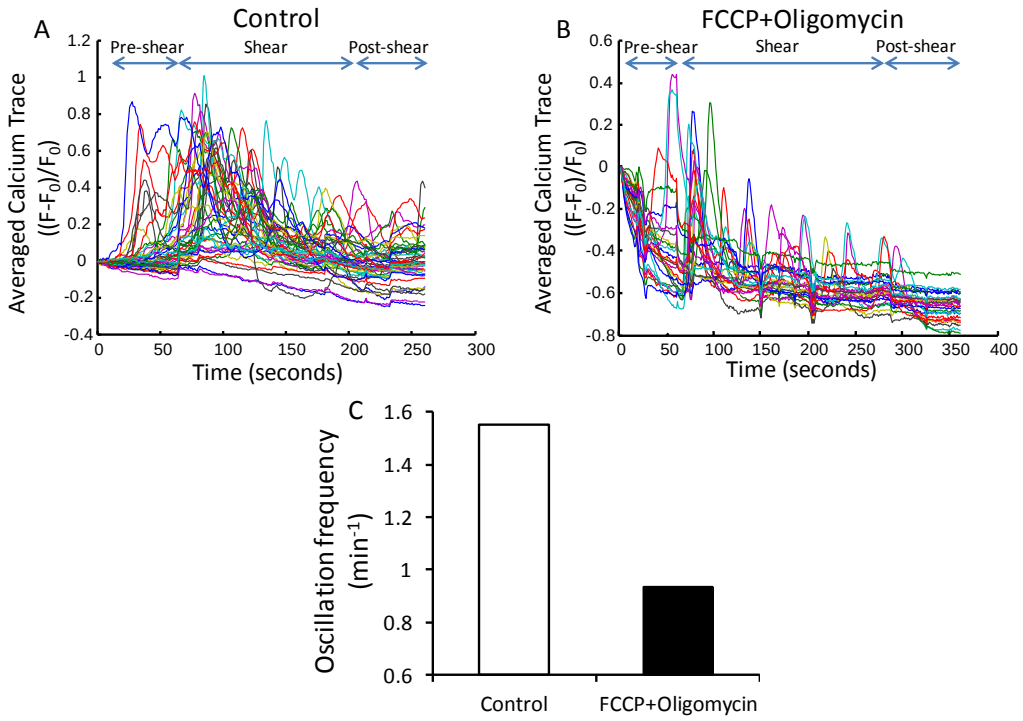

Figure 8. Role of mitochondria in shaping the shear-induced $[Ca^{2+}]_c$ response. (A) Cells exposed to steady laminar shear stress (10 dyne/cm^2) displayed $[Ca^{2+}]_c$ oscillations with respect to time. Each curve corresponds to the response of a single cell within the plane of view. (B) $[Ca^{2+}]_c$ response in cells treated with FCCP and oligomycin to inhibit the mitochondria. (C) Removing the buffering by the mitochondria altered the $[Ca^{2+}]_c$ signaling dynamics by decreasing the $[Ca^{2+}]_c$ oscillation frequency.

Chapter IV: Conclusion

As a fundamental component of I/RP injury, determining the effect of shear stress on $[Ca^{2+}]_c$ and mitochondrial Ca^{2+} signaling could have large implications in regards to treating this pathology. Through this work, we have found that when vascular endothelial cells are sheared, they produce $[Ca^{2+}]_c$ oscillations, and when NO is removed via the addition of L-NAME, the initial $[Ca^{2+}]_c$ transient is intensified and the oscillation frequency is decreased. This intensified $[Ca]_c$ transient could lead to mitochondrial dysfunction in these sheared cells. The goal of future work will be to delineate the mechanism which causes NO to have this affect. We will do this by strategically using chemical inhibitors of the sGC/cGMP/PKG pathways (**Figure 9**). In doing so, we will be able to determine if NO has any effect of on this pathway in endothelial cells, and if so, where in the pathway NO acts.

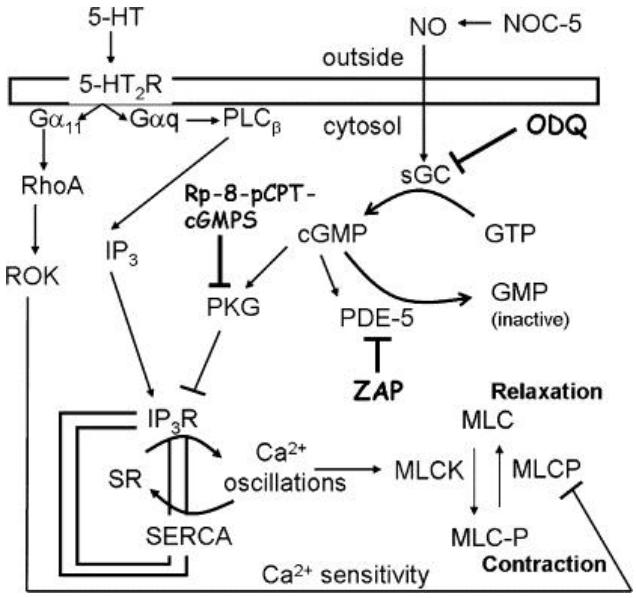

Figure 9. NO production could blunt the Ca^{2+} response in endothelial cells via the sGC/cGMP/PKG pathway. By upregulating this pathway, NO alters Ca^{2+} dynamics by indirectly inhibiting the IP_3R. Through the use of ODQ, ZAP, and Rp-8-pCPT-cGMPS we can determine where, if at all, NO alters the signaling in this pathway[14].

In addition, we will further determine the role of mitochondria on $[Ca^{2+}]_c$ signaling. We will do this through the use of specific mitochondrial toxins, such as FCCP/Oligomycin and antimycin (**Figure 10**). These experiments will first determine whether the mitochondria are, in fact, involved in $[Ca^{2+}]_c$ signaling dynamics. If this is proven true, then future experiments will also be performed to see exactly what role mitochondria play in these processes. Furthermore, we will study, through the use of compounds that inhibit components of the electron transport chain such as antimycin, the role of bioenergetics in the mitochondrial contribution to $[Ca^{2+}]_c$ signaling. I/RP alters the bioenergetics of mitochondria, so the information gained from these studies could give a greater understanding of the mechanisms that cause I/RP injury, and, in addition, could suggest therapeutic approaches to better treat this pathology.

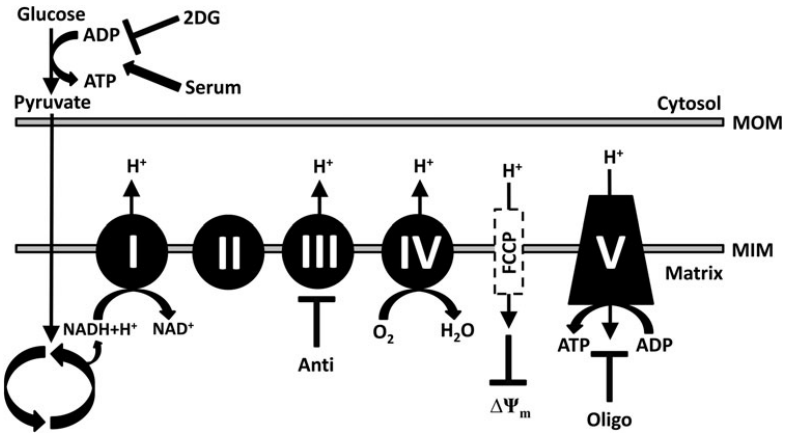

Figure 10. The primary components that form the mechanisms responsible for ATP production in endothelial cells. Glycolysis accounts for the majority of ATP produced in endothelial cells, and this process can be inhibited through the use of 2DG and accelerated with the addition of a nutrient rich serum. Complexes I, II, III, IV, and V lie on the inner mitochondria membrane with complex V (ATP synthase) actively utilizing the H^+ gradient to create ATP from ADP. The electron transport chain can be disturbed through the use of antimycin (complex III) inhibitor. The H^+ gradient can be dissipated by adding the uncoupler, FCCP, and ATP synthase activity can be inhibited by using oligomycin[13].

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